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Award Number: W81XWH-11-1-0406

TITLE: Development of a Tetrathioether (S4) Bifunctional Chelate System for Rh-105

PRINCIPAL INVESTIGATOR: Valerie Carroll

CONTRACTING ORGANIZATION: University of Missouri
Columbia, MO 65211-7600

REPORT DATE: July 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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| REPORT DOCUMENTATION PAGE | | | | Form Approved OMB No. 0704-0188 | |
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| 1. REPORT DATE (DD-MM-YYYY) July 2013 | | 2. REPORT TYPE Annual Summary | | 3. DATES COVERED (From - To) 15 June 2012 - 14 June 2013 | |
| 4. TITLE AND SUBTITLE Development of a Tetrathioether (S4) Bifunctional Chelate System for Rh-105 | | | | 5a. CONTRACT NUMBER W81XWH-11-1-0406 | |
| | | | | 5b. GRANT NUMBER W81XWH-11-1-0406 | |
| | | | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) Valerie Carroll, Don Wycoff, Gary Sieckman, Fabio Gallizzi, Timothy Hoffman, Silvia Jurisson | | | | 5d. PROJECT NUMBER | |
| | | | | 5e. TASK NUMBER | |
| | | | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Missouri Columbia, MO 65211-7600 | | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research Materiel Command Fort Detrick, MD 21701-5012 | | | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited | | | | | |
| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT We have previously, in the first year of this award, synthesized a new rhodium tetrathioether bombesin analogue, [Rh-S4-8Aoc-BBN(7-14)NH ₂] ⁺ . During the current period of investigation the molecule was tested for receptor binding affinity to the BB2 receptor on human prostate cancer PC-3 cells. Rhodium-105 radiolabeling studies were carried out with the S4-8Aoc-BBN(7-14)NH ₂ ligand system and also with other known chelate systems 3,3,3,3-S4-Diol, 3,3,3,3-S4-(COOH) ₂ . The [Rh-S4-8Aoc-BBN(7-14)NH ₂] ⁺ was found to exhibit a high affinity for the BB2 receptor (IC ₅₀ = 2.2 ± 0.3 nM) however high yields of the radiolabeled [¹⁰⁵ Rh-S4-8Aoc-BBN(7-14)NH ₂] ⁺ complex were not achieved under any conditions tested. To better understand these results, the 3,3,3,3-S4-Diol and 3,3,3,3-S4-(COOH) ₂ were investigated. It was revealed that traditional radiolabeling techniques for ¹⁰⁵ Rh are not compatible with systems that contain a carboxylic acid group. | | | | | |
| 15. SUBJECT TERMS Rhodium, Bombesin, Tetrathioether, Radiopharmaceutical, Targeted Radiotherapy | | | | | |
| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
| U | | | UU | 29 | USAMRMC |
| a. REPORT U | b. ABSTRACT U | c. THIS PAGE U | | | 19b. TELEPHONE NUMBER (include area code) |

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Introduction:

Rhodium-105 is an interesting candidate for radiopharmaceutical use due to its nuclear emissions ($\beta^- = 566$ keV; $\gamma = 319$ keV [19%], 306 keV [5%]) and kinetic stability. The goal of this project was to develop a new bombesin (BBN) targeted radiotherapeutic agent for ^{105}Rh using the bifunctional chelate technique. In pursuit of this objective, we have synthesized a new tetrathioether bombesin molecule, S4-8Aoc-BBN(7-14) NH_2 , which may have implications for prostate cancer therapy. We have also investigated the use of cyclic and acyclic tetrathioether chelates with ^{105}Rh and the implications this chelate choice may have on radiopharmaceutical development. This work adds to the current knowledge of ^{105}Rh ligand systems and will aid future researchers in the selection criteria for viable chelate systems.

Body:

Specific Aim 3: IC₅₀ Evaluation of the non-radioactive [RhCl-S4-8Aoc-BBN]⁺ complex

Rhodium tetrathioether bombesin, $[\text{RhCl-S4-8Aoc-BBN(7-14)NH}_2]^+$ was synthesized during the first year of this project. The previous annual report contains details of chelate synthesis, peptide coupling and formation of $[\text{RhCl-S4-8Aoc-BBN(7-14)NH}_2]^+$. The affinity of $[\text{RhCl-S4-8Aoc-BBN(7-14)NH}_2]^+$ for the gastrin releasing peptide receptor (type BB2) was evaluated using a competitive binding assay compared to $^{125}\text{I-Tyr}^4\text{-BBN}$ with BB2 receptor positive PC-3 human prostate cancer cells. In a micro-well plate approximately 3×10^5 PC-3 cells were suspended in Roswell Park Memorial Institute (RPMI) medium at pH 7.4 with 4.8 mg/mL HEPES, and 2 mg/mL BSA. The mixture was incubated at 37°C for 1 h with 30,000 cpm of $^{125}\text{I-Tyr-BBN}$ and increasing concentrations of $[\text{RhCl-S4-BBN(7-14)NH}_2]^+$ from 3.3×10^{-13} to 3.3×10^{-6} M. The cells were washed four times with media to release any non-specifically bound BBN and then counted on a Multi-Wiper (Laboratory Technologies, Maple Park, IL, USA) multiwell NaI gamma scintillation detector. The inhibitory concentration 50% (IC₅₀) was derived from the average of three experiments. The IC₅₀ curve (Figure 1) was obtained by plotting the % of $^{125}\text{I-}$

Tyr-BBN bound to the cell as a function of the concentration of $[\text{RhCl-S}_4\text{-BBN(7-14)NH}_2]^+$ added using GraphFit software version 4 (Erithacus Software Limited, Middlesex, UK).

The average concentration of $[\text{RhCl-S}_4\text{-8Aoc-BBN(7-14)NH}_2]^+$ needed to inhibit $^{125}\text{I-Tyr}^4\text{-BBN}$ by 50% (IC_{50}) was determined to be $2.2 \pm 0.3 \text{ nM}$. This value represents a significant improvement over the previously reported macrocyclic $\text{Rh-S}_4\text{-BBN(7-14)NH}_2$ ($\text{IC}_{50} = 37.5 \pm 10.5 \text{ nM}$) and $\text{Rh-S}_4\text{-5-Ava-BBN(7-14)NH}_2$ ($\text{IC}_{50} = 4.76 \pm 0.79 \text{ nM}$)^[1,2] and indeed is well within the range of recently investigated pre-clinical bombesin analogues for targeting prostate cancer.

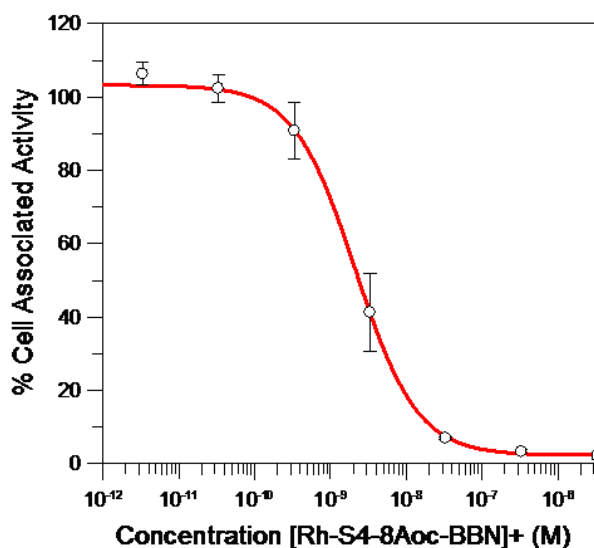


Figure 1: $[\text{RhCl-S}_4\text{-8Aoc-BBN(7-14)NH}_2]^+$ vs $^{125}\text{I-Tyr}^4\text{-BBN}$ competitive binding Assay.

Specific Aim 4: Radiolabeling of the S4-8Aoc-BBN(7-14)NH₂ chelate.

An ethanolic solution of S4-8Aoc-BBN(7-14)NH₂ was added to an aqueous solution of 0.5 - 1 mCi of rhodium-105 chloride at pH 3 - 4. Labeling conditions were varied from 2.5% - 57% ethanol and $5.8 \times 10^{-5} \text{ M}$ - $1.16 \times 10^{-3} \text{ M}$ S4-8Aoc-BBN(7-14)NH₂. In general, the following labeling conditions were investigated;

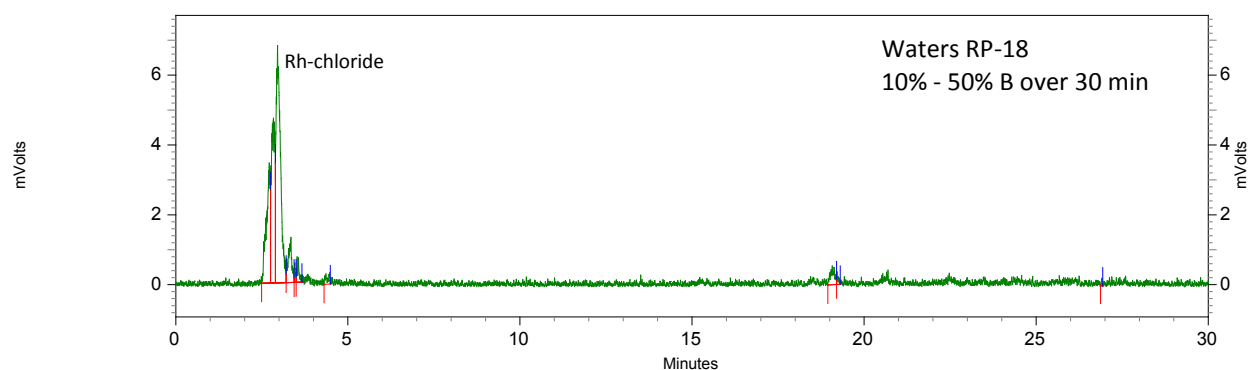
- 500 μL ($\sim 1 \text{ mCi}$) of Rh-105, 100 μL of 0.5 mg/mL S4-8Aoc-BBN(7-14)NH₂ in 15% ethanol.

Total ligand concentration: 5.8×10^{-5} M, Total ethanol: 2.5%

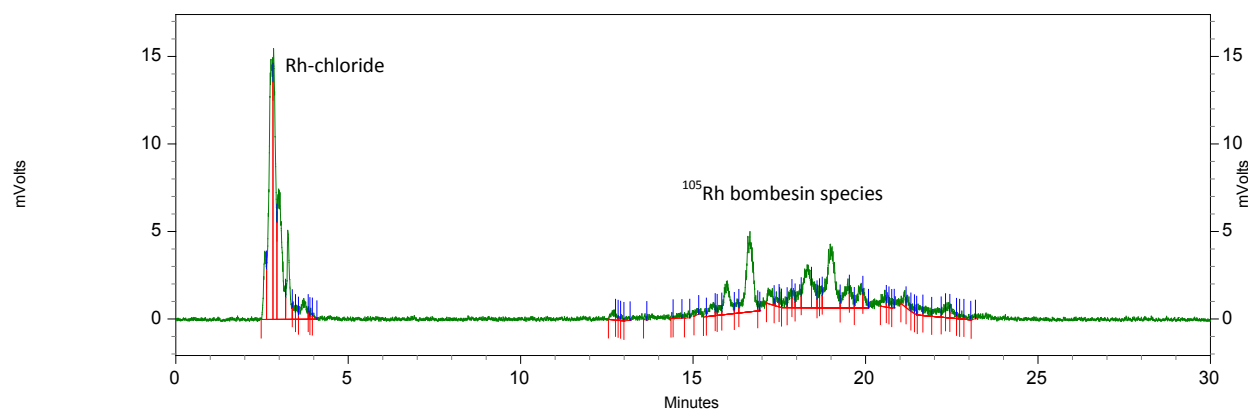
- 500 μ L (~ 1 mCi) of Rh-105, 100 μ L of 0.67 mg/mL S4-8Aoc-BBN(7-14)NH₂ in 15% ethanol.
Total ligand concentration: 7.8×10^{-5} M, Total ethanol: 2.5%
- 20 μ L (~ 50 μ Ci) of Rh-105, 200 μ L of 0.67 mg/mL S4-8Aoc-BBN(7-14)NH₂ in 15% ethanol.
Total ligand concentration: 4.2×10^{-4} M, Total ethanol: 13.6%
- 0.05 mg lyophilized S4-8Aoc-BBN(7-14)NH₂ in 50 μ L acetonitrile, 100 μ L ethanol
200 μ L (~ 1 mCi) of Rh-105 stock
Total ligand concentration: 9.46×10^{-4} M, Total ethanol: 28.5%
- 0.067 mg lyophilized S4-8Aoc-BBN(7-14)NH₂ in 50 μ L acetonitrile, 100 μ L ethanol
100 μ L (~ 0.5 mCi) of Rh-105 stock
Total ligand concentration: 1.84×10^{-4} M, Total ethanol: 40%
- 0.067 mg lyophilized S4-8Aoc-BBN(7-14)NH₂ in 50 μ L acetonitrile, 200 μ L ethanol
100 μ L (~ 1 mCi) of Rh-105 stock. (200 μ L was concentrated to 100 μ L at 90°C, N₂)
Total ligand concentration: 1.33×10^{-4} M, Total ethanol: 57.1%
- 0.5 mg lyophilized S4-8Aoc-BBN(7-14)NH₂ in 100 μ L acetonitrile, 100 μ L ethanol
100 μ L (~ 0.5 mCi) of Rh-105 stock
Total ligand concentration: 1.16×10^{-3} M, Total ethanol: 33%

After 1 h of heating at 80°C, all of these conditions resulted in low labeling yields (< 5 - 10%) as measured by analytical HPLC (Figure 2, a). Additional heating (85 °C, 2 h) resulted in formation of many radiolabeled species also observed by HPLC (Figure 2 b and c). The multiple products likely resulted from both hydrolysis of amide bonds on the peptide due to excess heat and from the existence of multiple ¹⁰⁵Rh-S4 species. For comparison, analytical HPLC chromatograms of non-radioactive S4-8Aoc-BBN(7-14)NH₂ (Figure 3, a) and [RhCl-S4-8Aoc-BBN(7-14)NH₂]⁺ (Figure 3, b) using the same solvent gradient, column and system are included below. To better understand the results we encountered with radiolabeling ¹⁰⁵Rh-S4-8Aoc-BBN(7-14)NH₂, the ¹⁰⁵Rh-S4 chemistry with cyclic 3,3,3,3-S4-Diol^[3] and acyclic 3,3,3-S4-(COOH)₂^[4-6] chelate systems was revisited.

(A) $^{105}\text{Rh-S4-8Aoc-BBN(7-14)NH}_2$ heated 1 h



(B) $^{105}\text{Rh-S4-8Aoc-BBN(7-14)NH}_2$ heated 2h



(C) $^{105}\text{Rh-S4-8Aoc-BBN(7-14)NH}_2$ heated 2h with cold RhCl_3 spike

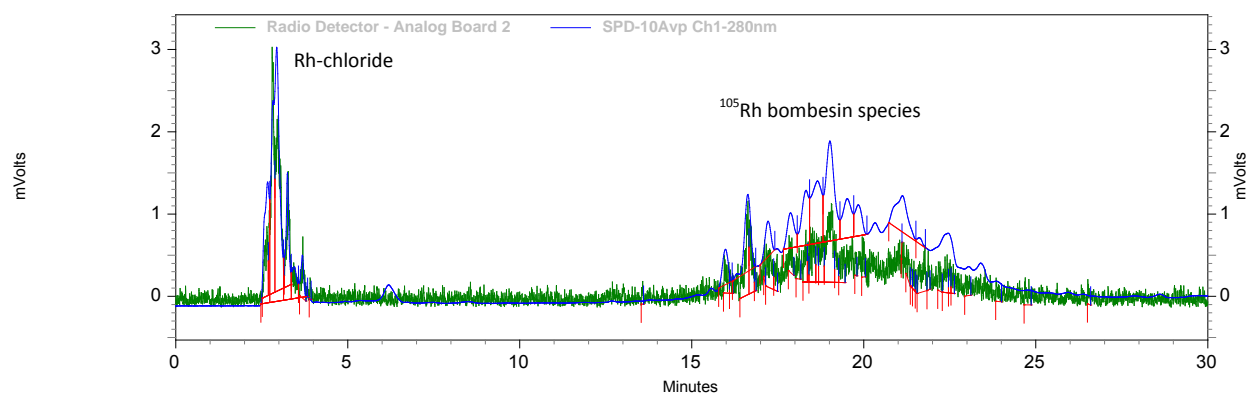
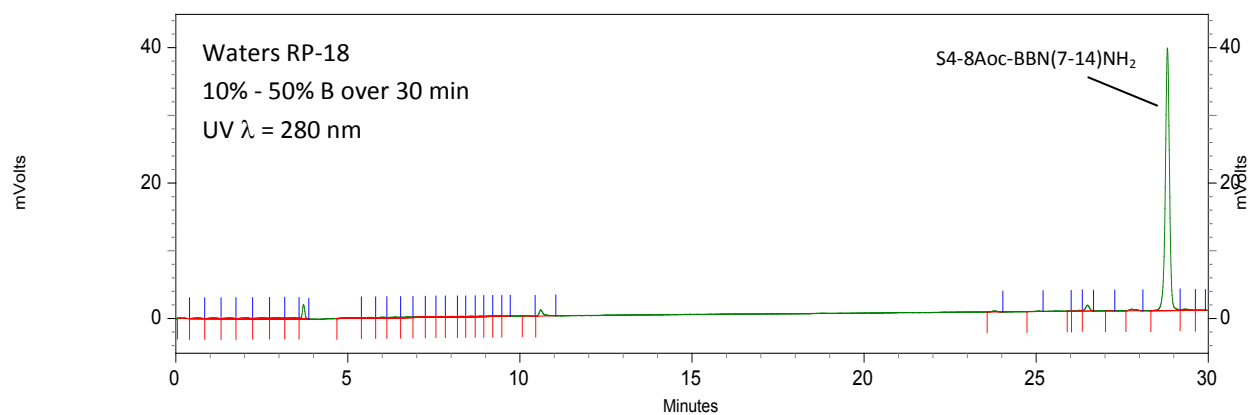


Figure 2: Representative HPLC radio chromatogram of $^{105}\text{Rh-S4-8Aoc-BBN(7-14)NH}_2$ for (a) 1 h heat at 80°C , (b) 2 h heat at 80°C , and (c) 2h heat with a spike of non-radioactive $\text{RhCl}_3 \cdot 3\text{H}_2\text{O}$.

(A) $3,3,3\text{-S4-8Aoc-BBN(7-14)NH}_2$



(B) $[\text{RhCl-S4-8Aoc-BBN(7-14)NH}_2]^+$

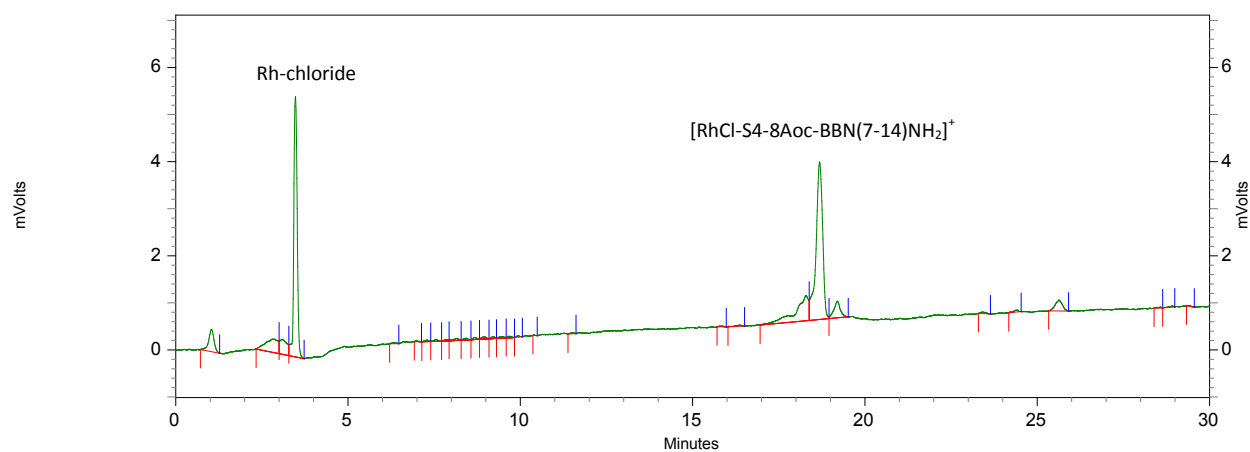


Figure 3: HPLC chromatogram of (a) $3,3,3\text{-S4-8Aoc-BBN(7-14)NH}_2$ and (b) $[\text{RhCl-S4-8Aoc-BBN(7-14)NH}_2]^+$ prepared on a macroscopic scale.

Investigation of the 3,3,3,3-S4-Diol chelate system

The macrocyclic 3,3,3,3-S4-Diol investigated by Venkatesh et al.^[3] is a well-known chelate for ¹⁰⁵Rh. This complex has been shown to provide >90% yields with ¹⁰⁵Rh via a quick labeling procedure and has previously been well characterized by silica gel TLC. Until recently 3,3,3,3-S4-Diol has been available commercially. For this reason many researchers studying new chelate systems for ¹⁰⁵Rh have used the ¹⁰⁵Rh-S4-Diol labeling procedure and analysis as a quick quality control procedure to determine the labeling efficiency of ¹⁰⁵Rhodium Chloride. We have elaborated on the previous TLC evaluation to include an HPLC method of evaluation. This new analysis has provided interesting results pertaining to labeling of thioether complexes with ¹⁰⁵Rh.

Non-radioactive RhCl₂-S4-Diol was prepared following the procedure reported by Venketesh^[3]. Briefly 0.80 mL (3.0 x 10⁻⁶ mol) of a 1.0 mg/mL solution of RhCl₃·3H₂O in acetonitrile was added to 1.0 mL (3.0 x 10⁻⁶ mol) of a 1.0 mg/mL solution of 3,3,3,3-S4-Diol in either 10% Ethanol/H₂O or 10% Ethanol/Saline at pH 4. The solution was heated at 80 °C for 1 h. Formation of chelated Rh-S4-Diol was confirmed by mass spectrometry. The macroscopic Rh-S4-Diol complex was evaluated by HPLC using a Waters Symmetry Shield RP-18 column (5 µm, 4.6 x 250 mm) with binary gradient where A is increased from 1% to 90% over 8 min, remains linear at 90% until 9 min and is decreased from 90% back to 1% by 10 min.

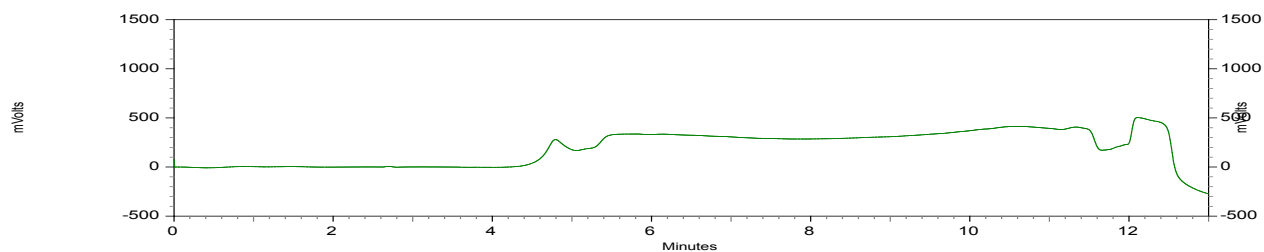
The radiolabeled ¹⁰⁵Rh-S4-Diol was synthesized according to the previously published procedure.^[22] Briefly, 100 µL (3.0 x 10⁻⁴ mol) of a 0.1 mg/mL solution of 3,3,3,3-S4-Diol in 15% Ethanol/H₂O was added to 500 µL (1 - 2 mCi) of ¹⁰⁵Rh Chloride at pH 4 and heated for 1 h at 80 °C. The resulting solution was spotted on a silica gel TLC plate and developed in 0.9% saline. The labeling solution was also evaluated using the HPLC method described above and compared to macroscopic results.

A 40 μL aliquot of the reaction mixture described above was also spiked with 20 μL (3.8×10^{-5} mol) of cold 1 mg/mL $\text{RhCl}_3 \cdot 3\text{H}_2\text{O}$ and heated for an additional hour at 80 $^\circ\text{C}$. Again this mixture was analyzed using HPLC allowing for in situ confirmation of radio chromatographic peaks with species observed via UV detection.

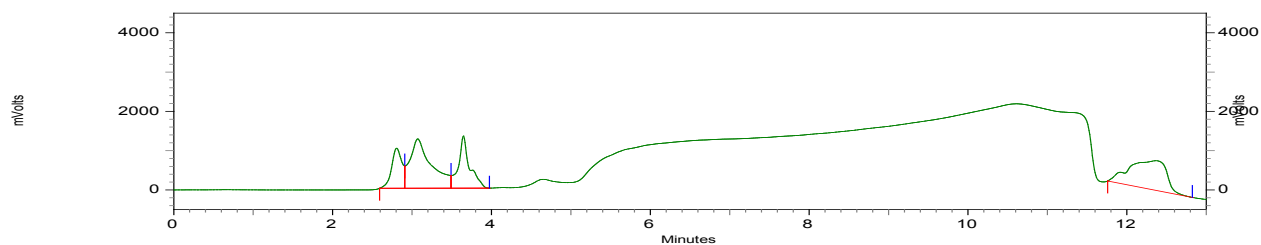
HPLC analysis of the non-radioactive Rh-S4-Diol complex prepared in a pH 3 aqueous ethanolic solution resulted in a single peak with a retention time of 5.65 min using a gradient of 1% B – 90% B over 8min (Figure 4, c). However analysis of the radiolabeled ^{105}Rh -S4-Diol exhibited two peaks under the same HPLC conditions, one at 5.81 min and a second peak at 5.53 min (Figure 4, d). A second non-radioactive Rh-S4-Diol complex was prepared in pH 3 ethanolic solution with excess NaCl. HPLC analysis of this solution revealed two peaks at 5.54 min and 5.80 min (Figure 4 e). ESI-MS evaluation (Figure 5) of the macroscopic solutions indicates the presence of both *trans*- $[\text{Rh}(\text{OH})_2\text{-S4-Diol}]^+$ ($m/z = 464.92$ Da, calc = 464.98 Da) (Figure 5, a) and *trans*- $[\text{RhCl}_2\text{-S4-Diol}]^+$ ($m/z = 500.97$ Da, calc = 500.91 Da) (Figure 4 b) based on the proposed structures (Figure 6).

This new data provides valuable information about the impact of reaction conditions on the species of *trans*- $\text{RhX}_2\text{-S4-Diol}$ formed and indicates that the radiotracer chemistry of ^{105}Rh complexes must be carefully evaluated for multiple isomers when halides are coordinated to the metal center. In the presence of a reducing agent such as ethanol, the coordinated halides are fairly labile and may be exchanged. Additionally, the HPLC method developed represents a new quantitative QC method for future researchers to analyze ^{105}Rh chloride labeling efficiency.

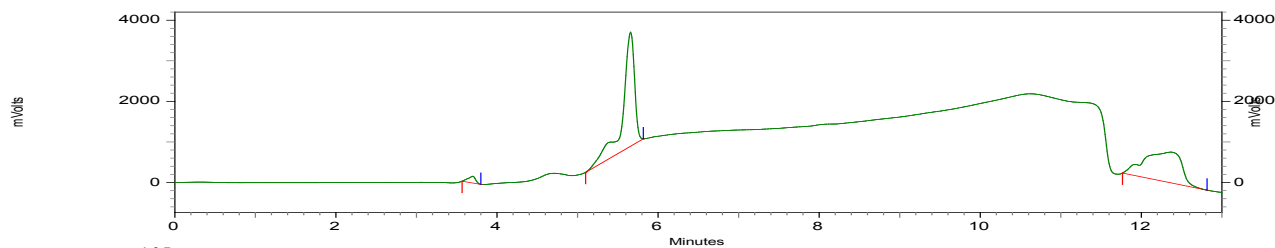
(A) Blank injection



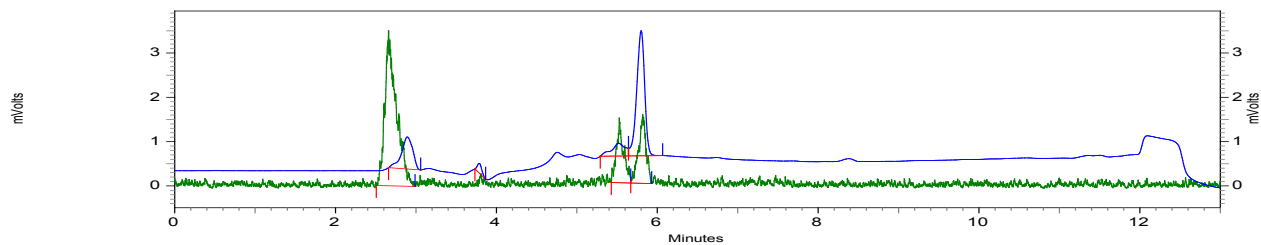
(B) Free Rhodium Chloride



(C) Rh-S4-Diol prepared in water



(D) ^{105}Rh -S4Diol with cold $[\text{Rh-S4}(\text{OH})_2\text{-Diol}]^+$ spike



(E) Rh-S4-Diol prepared in Saline

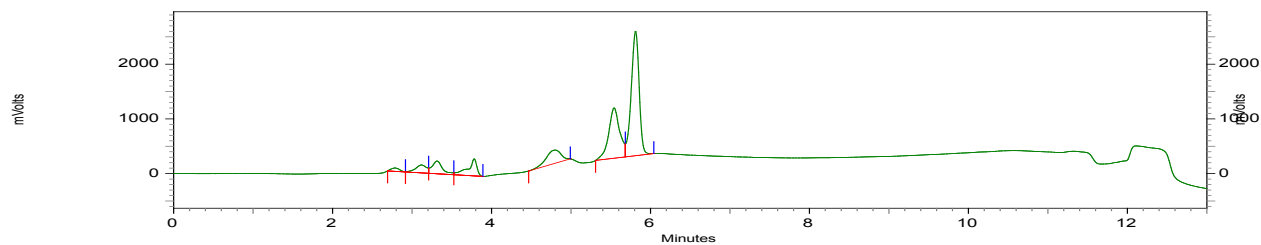


Figure 4: Summary of Rh-S4-Diol HPLC analysis.

B #1236-1720 RT: 4.66-6.49 AV: 485 NL: 9.90E3
T: ITMS + c ESI Full ms [350.00-2000.00]

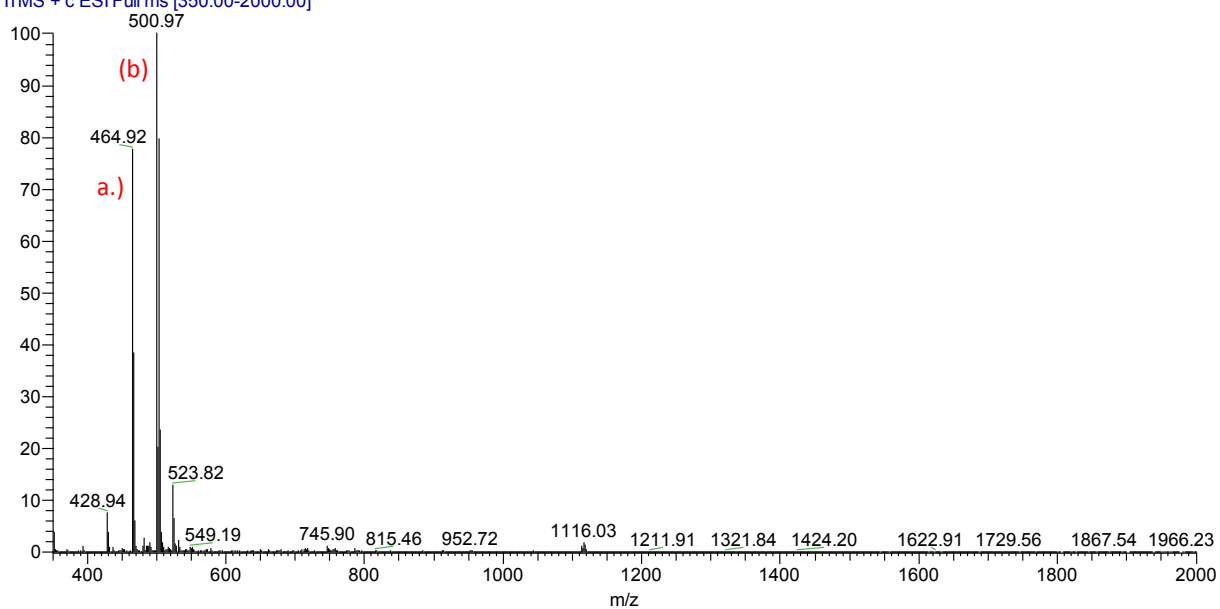


Figure 5: ESI-MS evaluation of Rh-S4-Diol prepared in saline.

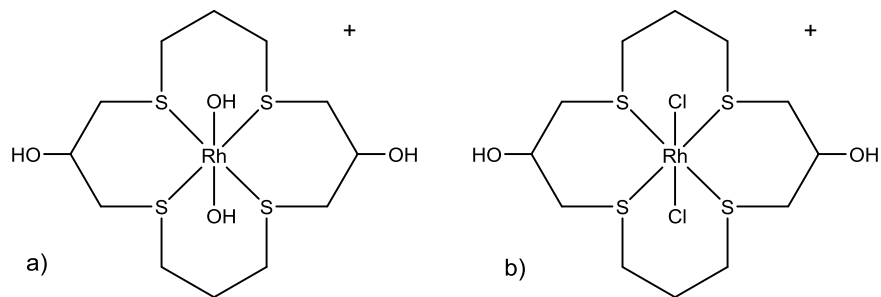


Figure 6: Macroscopic species, $\text{Rh}(\text{OH})_2\text{-S4-Diol}$ (a) and $\text{RhCl}_2\text{-S4-Diol}$ (b).

Investigation of the 3,3,3-S4-(COOH)₂ chelate system

The 3,3,3-S4-(COOH)₂ ligand system previously studied by Goswami et al.^[4-6] is most similar to our S4-8Aoc-BBN(7-14)NH₂ molecule. Using 3,3,3-S4-(COOH)₂ as a starting material, S4-8Aoc-BBN(7-14)NH₂ is formed by coupling the bombesin peptide to one of the carboxylate pendant groups on 3,3,3-S4-(COOH)₂. Goswami et al. reports formation of a single radiolabeled *trans*-[¹⁰⁵RhCl₂-S4-(COOH)₂]⁺ species as evaluated by silica gel thin layer chromatography (TLC) in which the product does not move from the origin; only ¹⁰⁵Rh chloride moves with the solvent front in saline.^[4-6] Based on the results observed for *trans*-RhX₂-S4-Diol, it was suspected that a number of Rh-S4-(COOH)₂ species were possible for this preparation as well and therefore a more quantitative analysis was performed using HPLC, mass spectrometry and NMR.

Non-radioactive Rh-S4-(COOH)₂ was prepared in ethanolic solutions with and without excess NaCl analogous to the Rh-S4-Diol preparation described above and analyzed by the same HPLC procedure. In the absence of excess NaCl the primary Rh(III) species formed is the mono chloride species, [RhCl-S4(COOH)(COO⁻)]⁺ with one coordinated pendant carboxylate group (m/z = 508.83 Da, calc = 508.92 Da) (Figure 7, a). This species is observed at an HPLC retention time of 6.14 min (Figure 8, b). When prepared with excess NaCl present the two species observed are *trans*-[RhCl₂-S4-(COOH)₂]⁺ (m/z = 544.93 Da, calc = 544.90 Da) (Figure 7, b) with a retention time of 6.87 min (Figure 8, c) and [RhCl-S4(COOH)(COO⁻)]⁺ with a retention time of 6.26 min (Figure 8, c). As previously discussed, in the presence of ethanol the coordinated chlorides are readily exchanged. If chloride ions (i.e., salt, NaCl) are not present in sufficient concentration, the coordinated chloride may exchange for another nearby donor atom, in this case a pendant carboxylate.

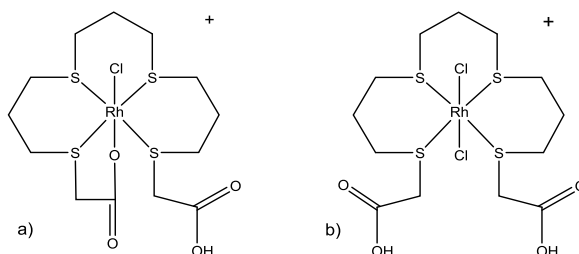
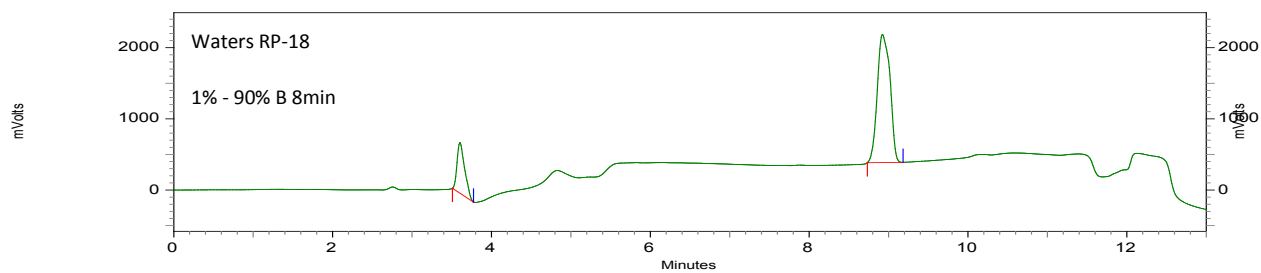
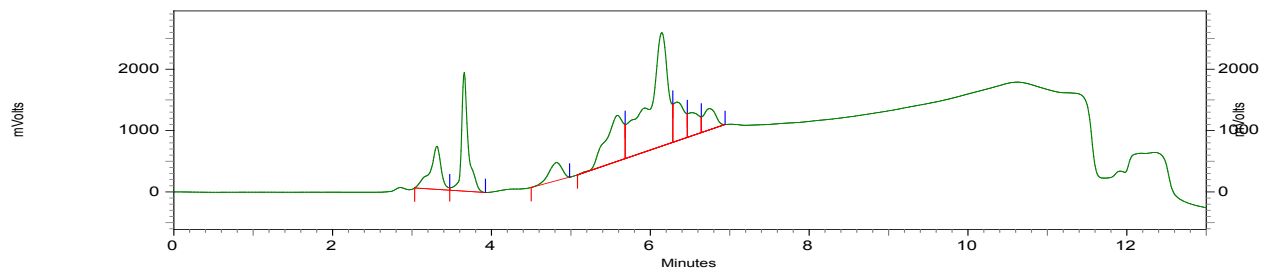


Figure 7: Initial species observed in initial macroscopic preparations.

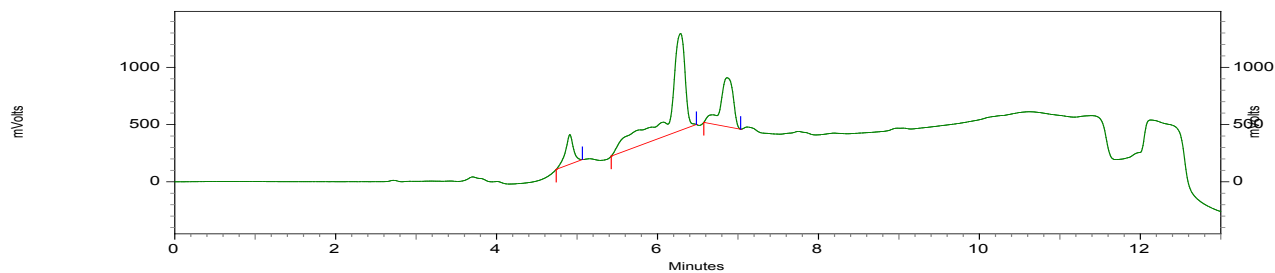
(A) 3,3,3-S4-(COOH)₂ Ligand



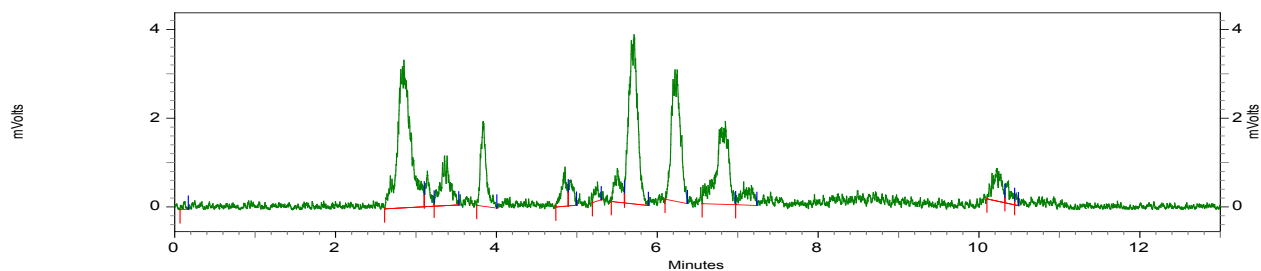
(B) RhX₂-S4-(COOH)₂ prepared no salt



(C) Rh-S4-(COOH)₂ prepared in saline



(D) ¹⁰⁵RhX₂-S4-(COOH)₂



(E) ¹⁰⁵RhX₂-S4-(COOH)₂ spiked with cold RhCl₃, heated 1h

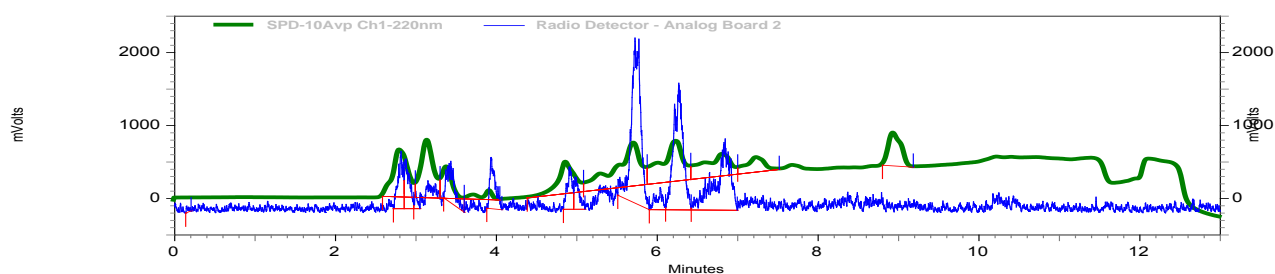


Figure 8: Summary of *trans*-RhX₂-S4-(COOH)₂ HPLC analysis

Since the existence of at least two *trans*-[RhX₂-(COOH)₂]⁺ species has been confirmed on a macroscopic scale, it is necessary to evaluate the radiotracer behavior. The ¹⁰⁵Rh chloride stock solution is in dilute HCl (pH ~ 1) following separation from the ¹⁰⁴Ru target material at the University of Missouri Research Reactor (MURR). Before labeling, this sample is adjusted to pH 3-4 with 0.1 M NaOH, which generates NaCl. We hypothesized that radiolabeling of the S4-(COOH)₂ ligand under these conditions would result in predominately the *trans*-[RhCl₂-S4-(COOH)₂]⁺ species. However HPLC analysis of the radiolabeling reaction mixture revealed at least 4 different peaks with retention times of 4.87 min, 5.71 min, 6.24 min and 6.82 min (Figure 8, d). The radiolabeled mixture was spiked with non-radioactive RhCl₃·3H₂O and heated for an additional hour. This test generates “carrier” Rh-S4-(COOH)₂ compounds in macroscopic amounts, which can be observed by UV (λ = 220 nm) to confirm a radiochromatographic peak and will highlight any differences between chemistry that occurs on the tracer level and chemistry that occurs on the macroscopic level under the same conditions. The UV trace confirmed all 4 of the tracer peaks observed (Figure 8, e). It is clear that under these conditions it is possible to make multiple Rh-S4-(COOH)₂ species.

At this point, we hypothesized that the additional peaks by HPLC may be due to any combination of dichloro, dihydroxo, and pendant carboxylate coordinated *trans*-RhX₂-S4-(COOH)₂ species.

During the initial macroscopic evaluation of *trans*-[RhX₂-S4-(COOH)₂]⁺ two species were observed where either X₂ = Cl₂ (t_r = 6.87 min) or X₂ = Cl, pendant COO⁻ (t_r = 6.14 min) depending on the concentration of NaCl present in solution. Using the published radiolabeling conditions^[4,6] more than two *trans*-[¹⁰⁵RhX₂-S4-(COOH)₂]⁺ species were observed by HPLC (t_r = 4.87 min, 5.71 min, 6.24 min and 6.82 min). It was suspected that the *trans*-[Rh(OH)₂-S4-(COOH)₂]⁺ species might also be possible in a manner analogous to the observed *trans*-[Rh(OH)₂-S4-Diol]⁺ when water is present. Therefore reaction conditions varying the amount of water present and the amount of salt present were investigated.

In order to identify as many Rh-S4-(COOH)₂ species as possible, the complex was formulated under a series of reaction conditions all at pH 3: (1) all organic solvent reaction conditions (50% ethanol/acetonitrile), (2) an organic solvent system with excess NaCl (saturated), (3) an aqueous-organic solvent mixture (25% water, 25% ethanol, 50% acetonitrile) and (4) an aqueous solution with excess NaCl (0.1 g). All reaction mixtures were heated for 1 h at 80°C, cooled, and then analyzed using the same HPLC method described above. The reaction mixtures were then lyophilized and taken up in either 50% acetonitrile/water for ESI-MS evaluation or 50% d₃-acetonitrile/D₂O for NMR studies.

HPLC evaluation (Waters RP-18, 1% B – 90% B over 8 min) of sample (1) prepared in 50% ethanol/acetonitrile resulted in a primary peak with a retention time of 6.20 min. Sample (2) prepared in 50% ethanol/acetonitrile saturated with NaCl resulted in a primary peak with a retention time of 6.19 min. The solubility of NaCl in ethanol and acetonitrile is low, 0.65 g/kg and 0.003 g/kg respectively. Thus the conditions in these two preparations are quite similar and similar HPLC results are not surprising.

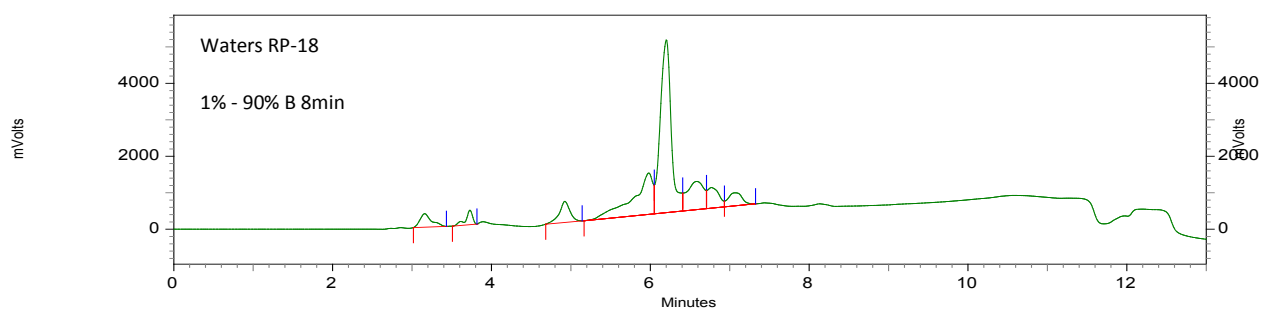
The macroscopic synthesis of *trans*-[RhCl₂-S4-(COOH)₂]⁺ previously reported^[4,5] was carried out in acetonitrile/ethanol solution. The radiotracer synthesis is carried out quite differently because of the aqueous starting solution available for ¹⁰⁵Rh-chloride following separation from its target. Our macroscopic preparation of samples (3) prepared in 25% water, 25% ethanol, 50% acetonitrile and (4) prepared in 25% water, 25% ethanol, 50% acetonitrile with 0.1 g NaCl are more similar to radiolabeling conditions.

HPLC evaluation of sample (3) prepared in 25% water, 25% ethanol, 50% acetonitrile resulted in a broad peak (or group of overlapping peaks) with retention times of ~ 5.06 – 6.54 min. Within this region two significant peaks are observed with retention times of 5.68 min and 6.21min. Under these conditions it appears that many species are formed.

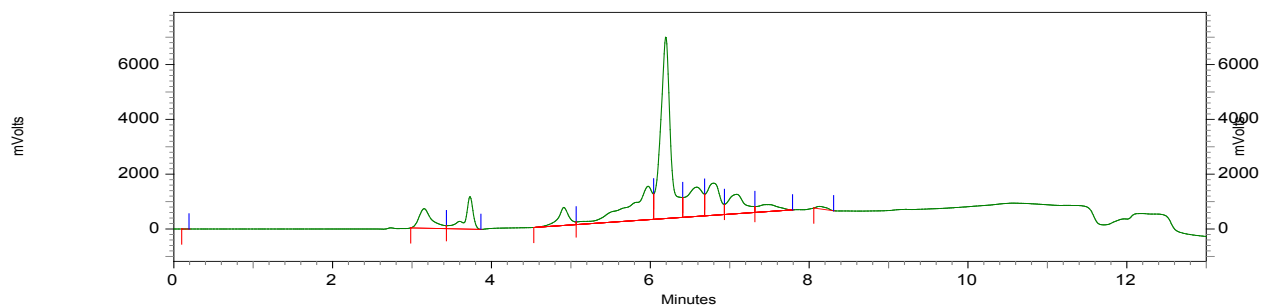
Evaluation of sample (4) prepared in 25% water, 25% ethanol, 50% acetonitrile with 0.1 g NaCl resulted in a primary peak with a retention time of 6.18 min. This result suggests that addition of excess NaCl may be used to encourage formation of a single predominate species.

The amount of NaCl present during radiotracer formulation of *trans*-[$^{105}\text{RhX}_2\text{-S4-(COOH)}_2$] $^+$ is currently unknown. During separation of ^{105}Rh from $^{104/105}\text{Ru}$, significant amounts of NaCl are generated by the addition of HCl to a mixture of NaOCl and NaOH. The NaOCl was generated *in situ* by bubbling of Cl_2 gas into NaOH. Since it is unknown how much NaOCl is generated at this step, the amount of NaCl generated by addition of acid to the separation solution is also unknown. It is possible that the concentration of NaCl present in our radiolabelling formulation is less than the amount present (0.025 mg/mL) in sample (4). Addition of more salt to the radiolabeling solution may improve the yield of a single (or more predominate) species.

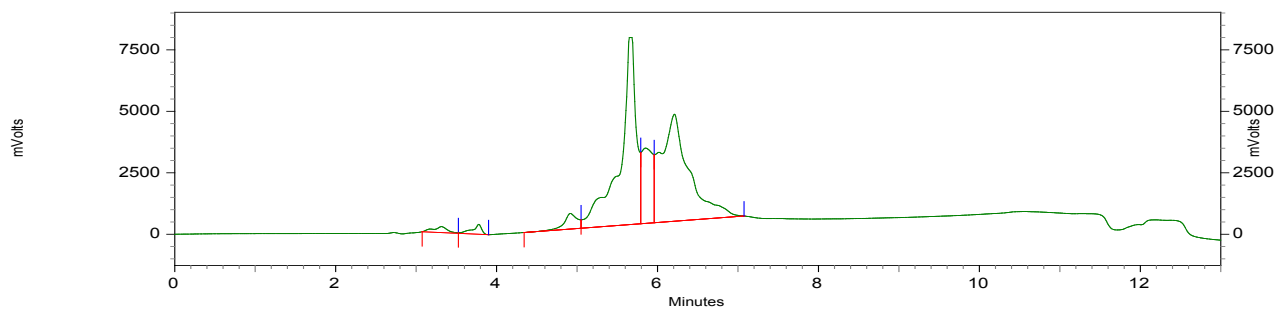
Sample (1) 50% Ethanol/Acetonitrile



Sample (2) 50% Ethanol/Acetonitrile + NaCl



Sample (3) 25% Water/25% Ethanol/50% Acetonitrile



Sample (4) 25% Water/25% Ethanol/50% Acetonitrile + NaCl

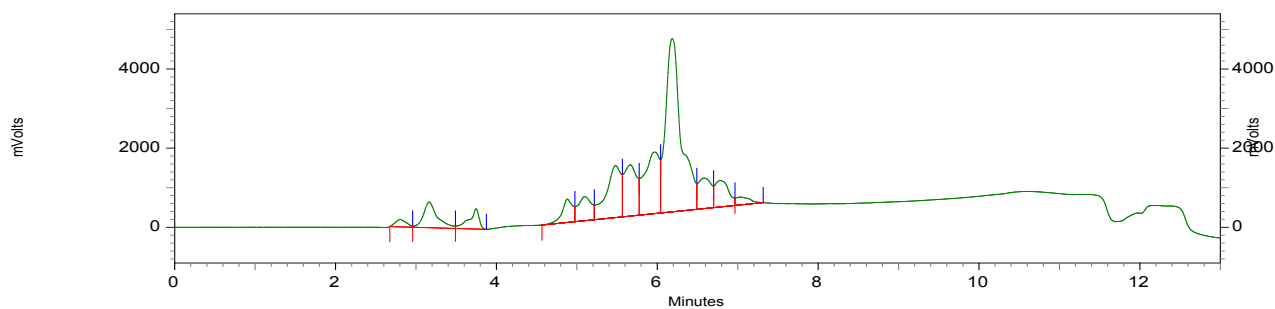
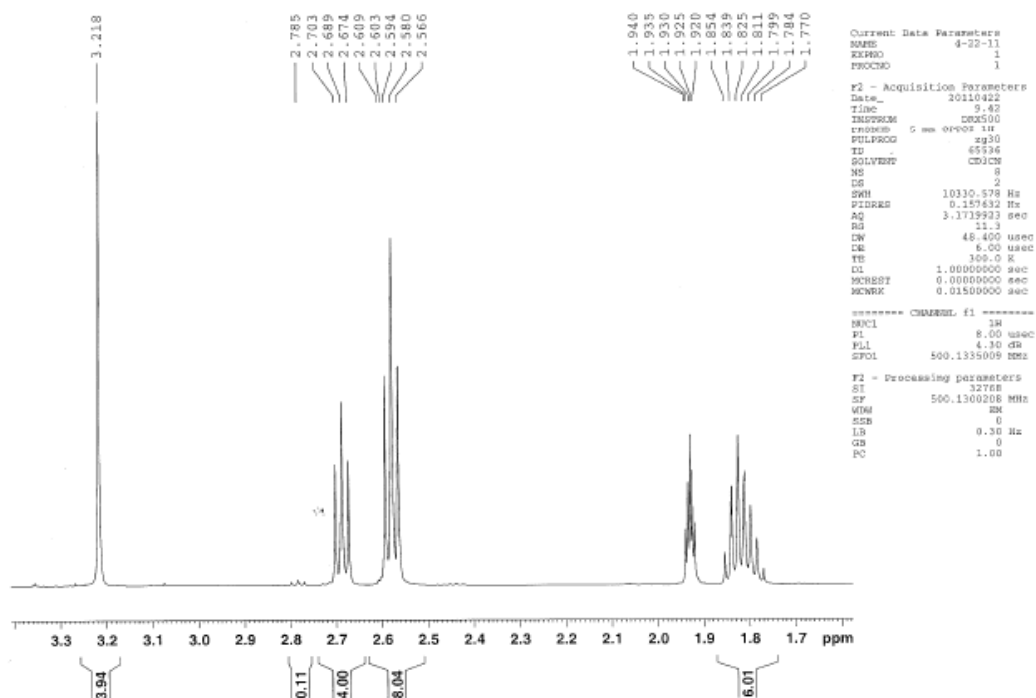
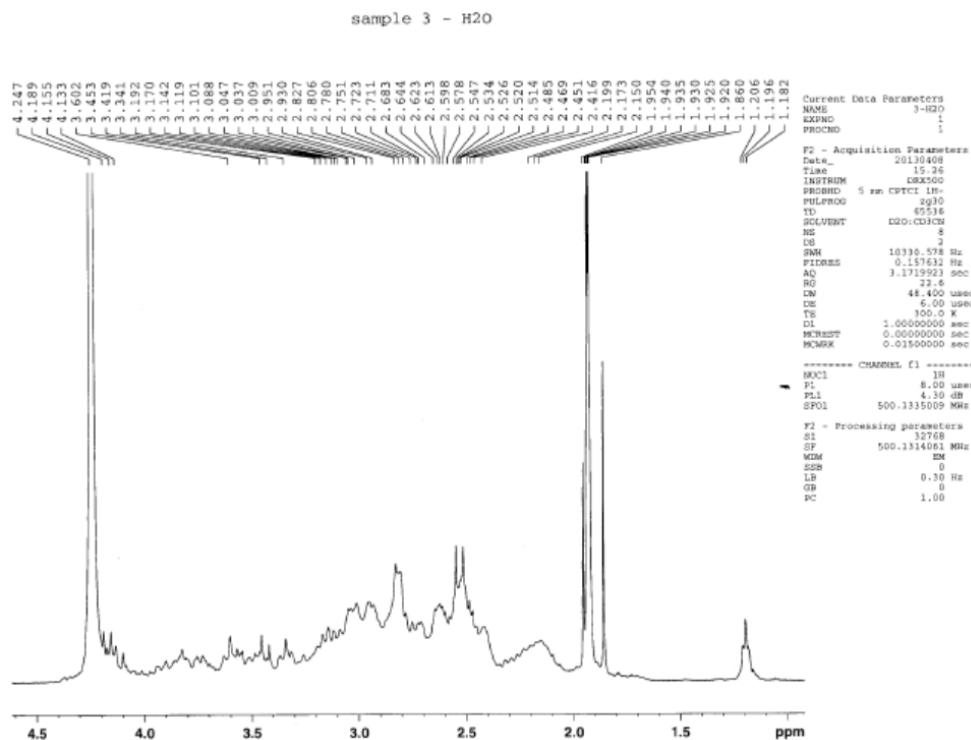


Figure 9: HPLC analysis of samples prepared under reaction conditions 1-4.

The lyophilized *trans*-[RhX₂-S4-(COOH)₂]⁺ was taken up in d₃-acetonitrile and evaluated by NMR. The presence of multiple isomers of the product will result in a complex spectrum with overlapping peaks, especially in the regions for the three propylene backbone protons. However, the methylene group on the terminal thioethers should be observed as singlets if the carboxylate group is not coordinated and a doublet of doublets (each proton unique) if it is coordinated to the Rh center.

Based on ¹HNMR and COSY analysis it is evident that multiple Rh-S4-(COOH)₂ species are present in each of the samples. 3,3,3-S4-(COOH)₂ is a symmetrical molecule. Each H is chemically equivalent to the corresponding H in the other half of the molecule (across the plane of symmetry). Thus the ¹HNMR spectrum of the uncomplexed 3,3,3-S4-(COOH)₂ chelate shows relatively few peaks (Figure 10). Upon complexation with Rh chloride, if there were only one *trans*-RhCl₂-S4-(COOH)₂ species produced with a single isomer, as previously believed, one would not expect any increase in the number of peaks observed, only a change in chemical shifts. ¹HNMR evaluation of the RhX₂-S4-(COOH)₂ complexes formed when prepared in aqueous solutions results in the appearance of many additional peaks. This is indicative of the presence of multiple RhX₂-S4-(COOH)₂ species (Figure 11), some of which may involve coordination to a pendant carboxylate group. Species with a coordinated carboxylate do not have a plane of symmetry. Each H on the molecule is chemically unique, and therefore a more complex spectrum (many more peaks) can be expected.

It is not possible to identify any species based on this ¹HNMR evaluation, but it is clear that multiple species are present. The multiple overlapping peaks can be explained by either formation of additional *trans*-RhX₂-S4-(COOH)₂ species (all differing in X₂ coordinated atoms) and/or the existence of more than one isomer of *trans*-RhX₂-S4-(COOH)₂.

Figure 10: ^1H NMR of 3,3,3-S4-(COOH) $_2$ ligand.Figure 11: ^1H NMR analysis of $\text{trans-}[\text{RhX}_2\text{-S4-(COOH)}_2]^+$ prepared in an aqueous solution.

Using ESI-MS several compounds were identified based on proposed structures as summarized in Figure 12 and Table 1. Samples employing reaction conditions (1) and (2) were very similar. Both were prepared in 50% ethanol/acetonitrile, sample (2) with the addition of excess NaCl. Under these conditions, $\text{RhCl}_2\text{-S4-(COOH)}_2$ ($m/z = 544.91$ Da, $\text{calc} = 544.90$ Da) (Figure 12 a) and $\text{RhCl}_2\text{-S4-(COOH)(COOEt)}$ ($m/z = 572.89$ Da, $\text{calc} = 572.93$ Da) (Figure 12 b) were predominant and to a somewhat lesser extent $\text{RhCl-S4-(COOH)(COO-)}$ where a pendant carboxylate group is coordinated to the metal center ($m/z = 508.87$ Da, $\text{calc} = 508.92$ Da) (Figure 12 d) is also observed (Figures 13 and 14). The ethyl ester (Figure 12, b) is formed by acid catalyzed esterification in the presence of ethanol.

In sample (4) under aqueous conditions with excess NaCl the formation of $\text{RhCl}_2\text{-S4-(COOH)}_2$ ($m/z = 544.91$ Da, $\text{calc} = 544.90$ Da) (Figure 12 a) is dominant. However small amounts of $\text{RhCl-S4-(COOH)(COO-)}$ ($m/z = 508.87$ Da, $\text{calc} = 508.92$ Da) (Figure 12 d), $\text{RhCl}_2\text{-S4-(COOH)(COOEt)}$ ($m/z = 572.89$ Da, $\text{calc} = 572.93$ Da) (Figure 12 b) and $\text{RhCl}_2\text{-S4-(COOEt)}_2$ ($m/z = 600.91$ Da, $\text{calc} = 600.96$ Da) (Figure 12 c) are also present (Figure 16).

The presence of all five species was observed in sample (3) prepared in aqueous conditions (25% water, 25% ethanol, 50% acetonitrile no salt). A significant yield of all three species $\text{RhCl}_2\text{-S4-(COOEt)}_2$ ($m/z = 600.91$ Da, $\text{calc} = 600.96$ Da) (Figure 12 c), $\text{RhCl-S4-(COOH)(COO-)}$ ($m/z = 508.87$ Da, $\text{calc} = 508.92$ Da) (Figure 12 d), and $\text{RhCl-S4-(COOEt)(COO-)}$ ($m/z = 536.90$ Da, $\text{calc} = 536.95$ Da) (Figure 12 e) was observed (Figure 15). This is the only sample in which the monochloro pendant carboxylate coordinated $\text{RhCl-S4-(COOEt)(COO-)}$ species was observed with a pendant ethyl ester. We had hypothesized that a $\text{Rh(OH)}_2\text{-S4-(COOH)}_2$ species analogous to the observed $\text{Rh(OH)}_2\text{-S4-Diol}$ may be present in aqueous solutions without NaCl, however this species was not observed.

There is a significant difference between sample (3), which was prepared in a solution of 25% water, 25% ethanol, 50% acetonitrile, and sample (4), which was prepared in the same solution with the

addition of 0.1 g NaCl. Without the presence of excess chloride ions in solution the pendant coordinated monochloride species $\text{RhCl-S4-(COOH)(COO-)}$ and $\text{RhCl-S4-(COOEt)(COO-)}$ are favored in addition to other species. With the presence of excess chloride ions in solution the dichloro species especially $\text{RhCl}_2\text{-S4-(COOH)}_2$ are more favored. The solvents used in radiolabeling are aqueous and it is likely that the concentration of NaCl present during radiolabeling is in between the conditions in samples (3) and (4). Therefore the addition of more NaCl might favor the production of a more dominate single $^{105}\text{RhX}_2\text{-S4-(COOH)}_2$ species.

The pendant ethyl esters on species (b), (c) and (e) (Figure 12) are formed by acid catalyzed Fischer esterification of the pendant carboxylic acid. An acidic solution is required to prevent the formation of $^{105}\text{Rh(OH)}_3$ and ethanol is required as a reducing agent. If neither acid nor ethanol can be eliminated, the formation of ethyl esters will continue to be a competing reaction for this formulation. The results of this study indicate that traditional radiolabeling techniques used for ^{105}Rh are not compatible with molecules that contain carboxylic acid groups.

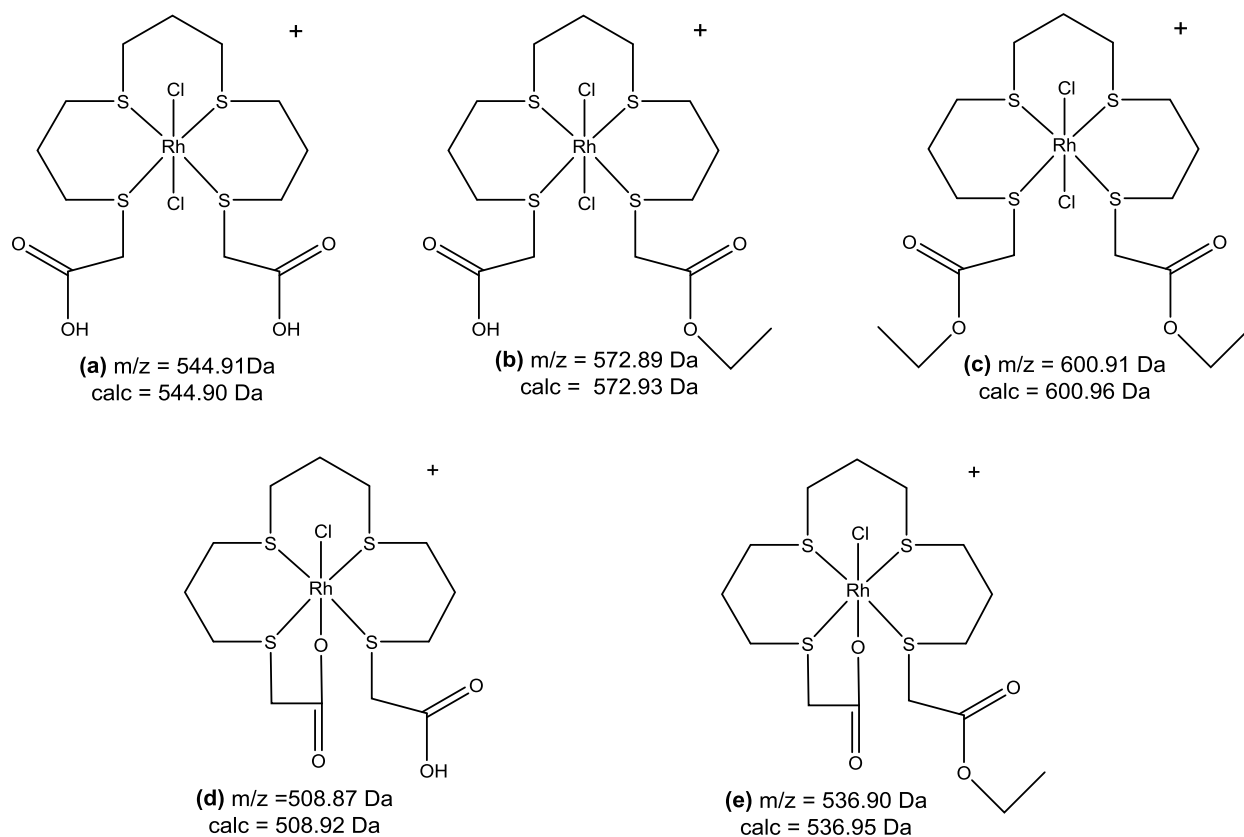


Figure 12: Rh-S4-(COOH)₂ species identified in samples 1-4.

| Sample | Reaction Conditions | Products observed |
|--------|---|----------------------|
| 1 | 50% ethanol/acetonitrile | a, b, d |
| 2 | 50% ethanol/acetonitrile + NaCl | a, b, d |
| 3 | 25% water, 25% ethanol, 50% acetonitrile | a, b, c, d, e |
| 4 | 25% water, 25% ethanol, 50% acetonitrile + NaCl | a, b, c, d |

Table 1: Products observed for various reaction conditions

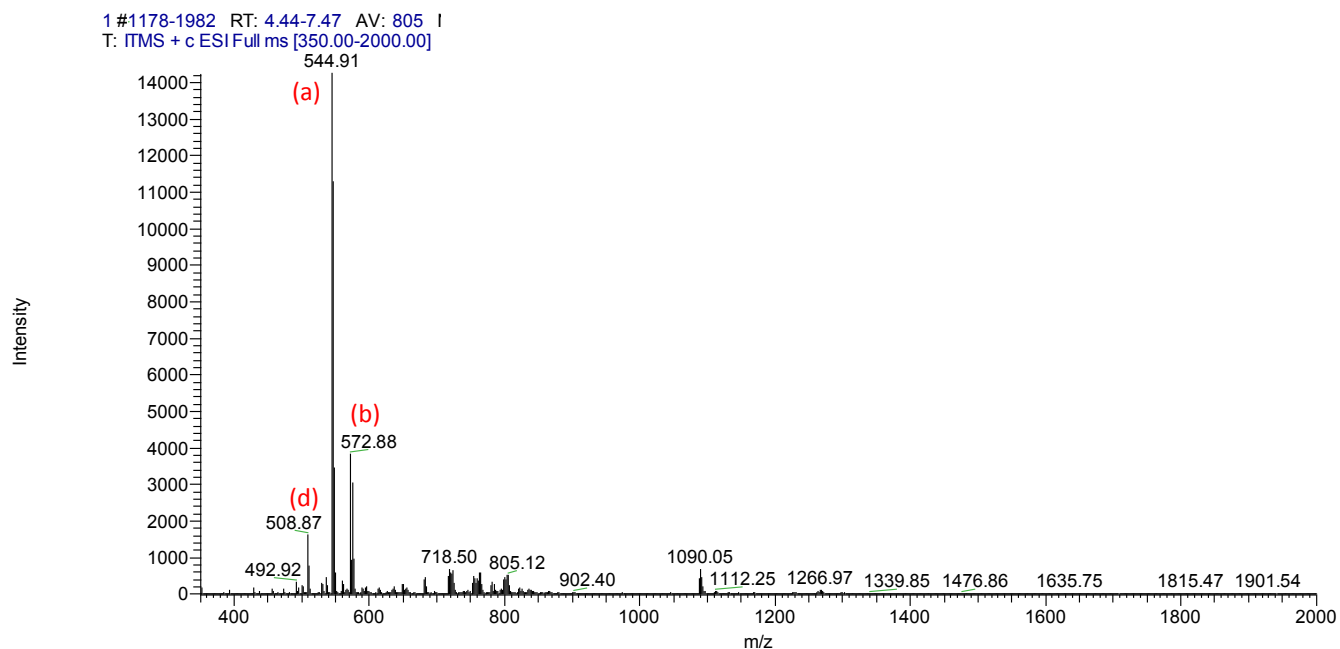


Figure 13: Rh-S4-(COOH)₂ Sample (1) prepared in 50% ethanol/acetonitrile.

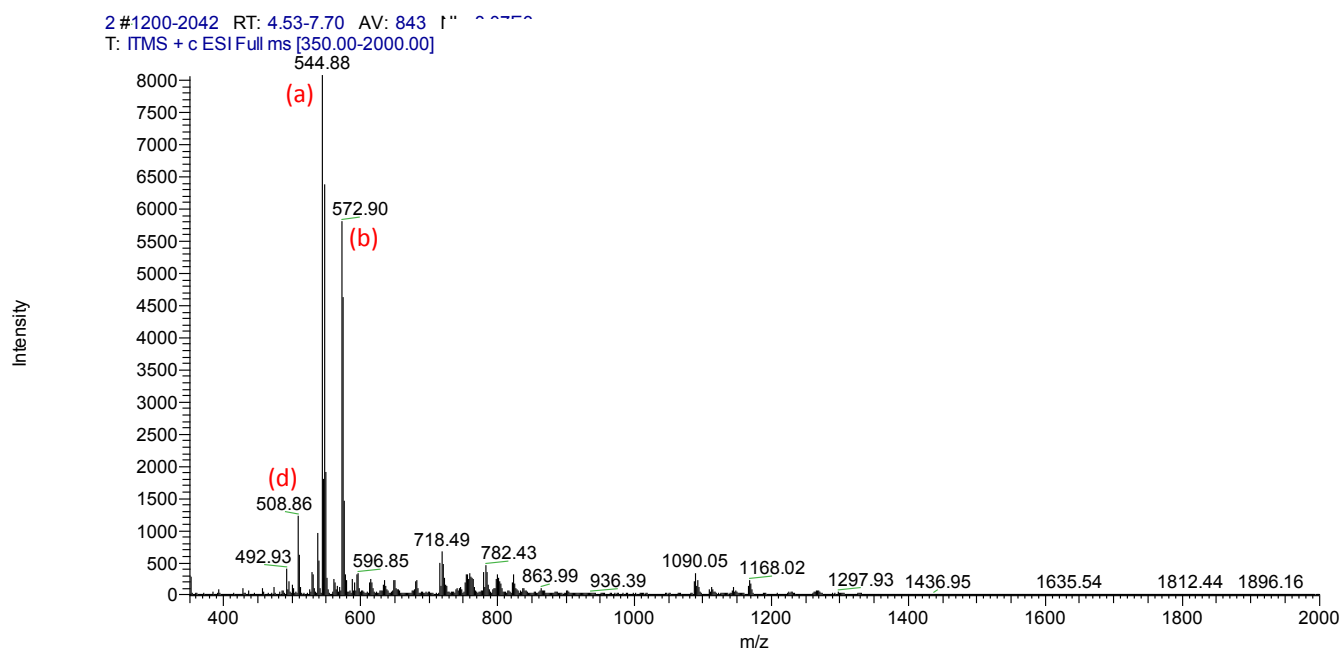


Figure 14: Rh-S4-(COOH)₂ Sample (2) prepared in 50% ethanol/acetonitrile + NaCl.

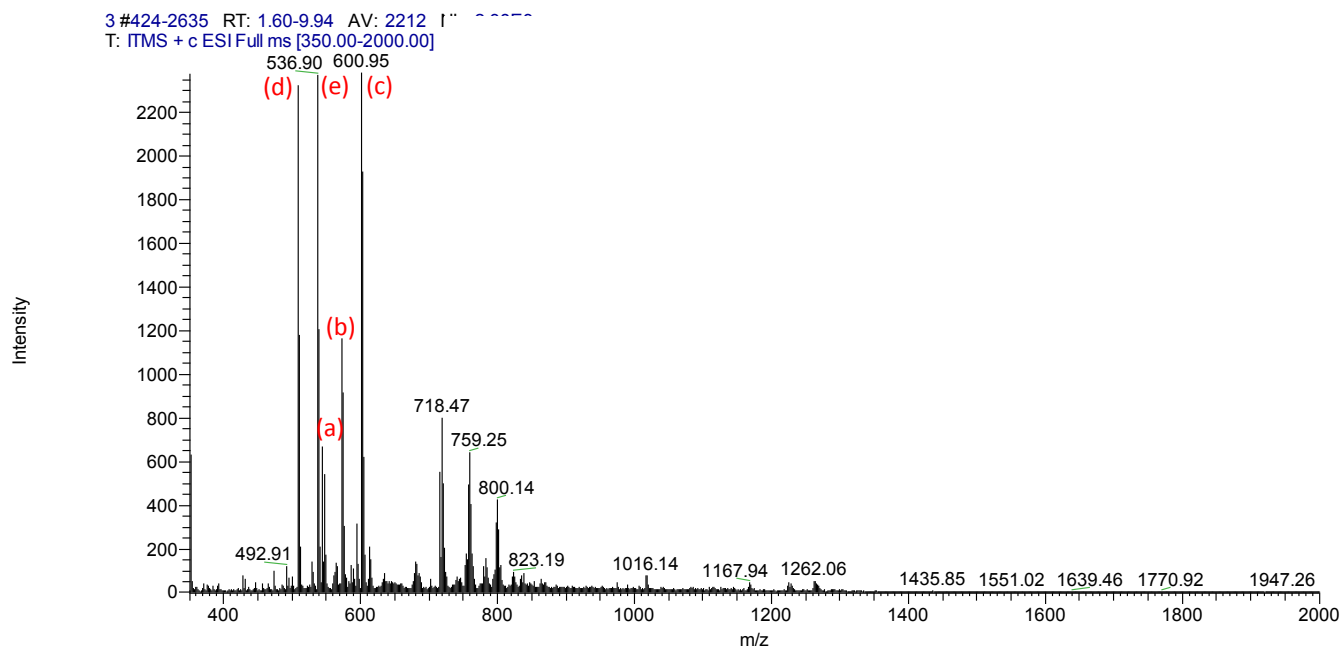


Figure 15: Rh-S4-(COOH)₂ Sample (3) prepared in 25% water, 25% ethanol, 50% acetonitrile.

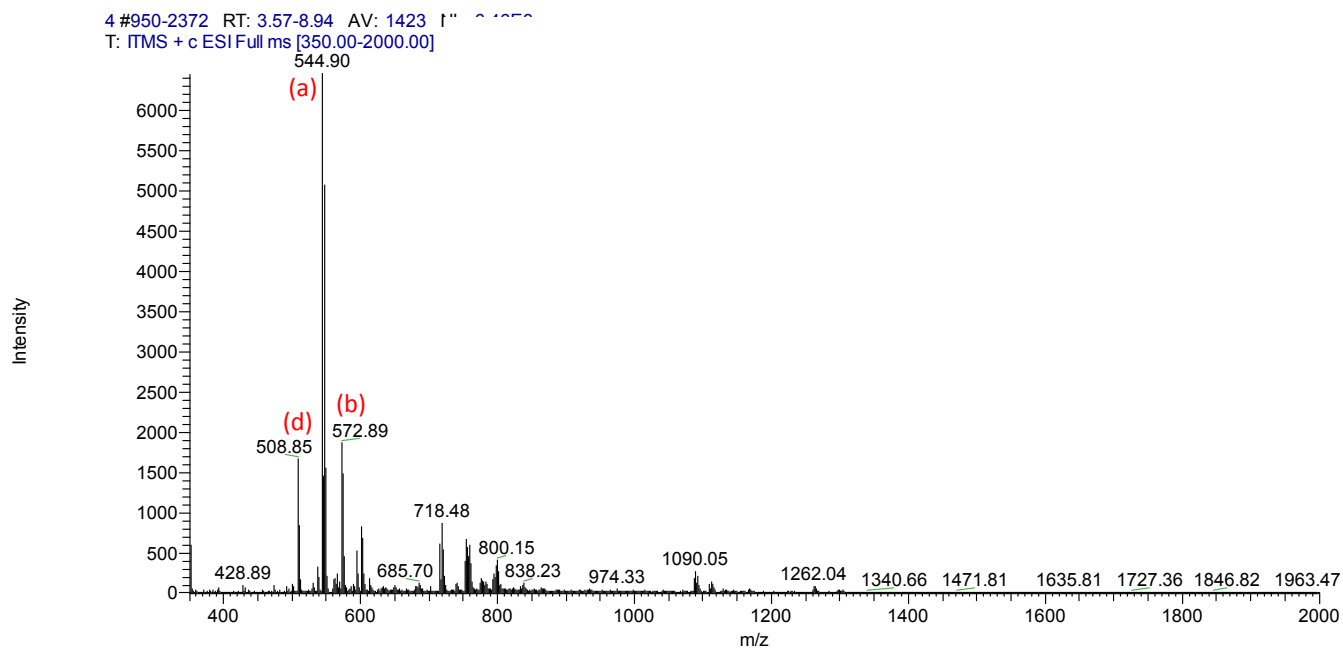


Figure 16: Rh-S4-(COOH)₂ Sample (4) prepared in 25% water, 25% ethanol, 50% acetonitrile + NaCl.

Specific Aim 5: Animal biodistribution studies

Animal biodistribution studies could not be carried out since a high yield of a single conformer of $[^{105}\text{RhX}_2\text{-S4-8Aoc-BBN(7-14)NH}_2]^+$ could not be obtained. Indeed recent studies indicate that formation of a single species cannot be formed with any chelate that contains a carboxylic acid group using traditional radiolabeling techniques for ^{105}Rh .

Specific Aim 6: Present and publish results

This work has been published in the form of a Ph.D. thesis and has also been prepared in paper form to be submitted for publication in the near future.

Training Program

Over the past year this grant has provided me with many opportunities to develop my career. In the fall semester I took Biochem 4460 – Cancer Biology in addition to my previously completed course work in chemistry. This class provided me with additional material to diversify my training as a translational scientist in the field of Cancer Research. This year, and throughout my graduate studies, I have attended symposia offered by the University of Missouri Chemistry Department and the Radiopharmaceutical Sciences Institute. I have benefited from one on one mentoring with leaders in the field of Radiopharmaceutical Chemistry, Dr. Silvia Jurisson and Dr. Timothy Hoffman. I successfully completed my degree in May 2013, earning a Ph.D. in chemistry based on research supported by this grant. I have accepted a postdoctoral position with Dr. David Wilson at University of California San Francisco, where I plan to continue my work as a prostate cancer researcher focusing on the development of redox sensitive positron emission radiotracers.

Key Research Accomplishments:

- Synthesized a new rhodium tetrathioether molecule with high affinity for the BB2 receptor on human prostate cancer PC-3 cells

- Developed a new quantitative QC method for analyzing radiolabeling efficiency of rhodium-105
- Analyzed the effect of chloride ion concentration in labeling solutions of final radiolabeled ^{105}Rh tetrathioether products for cyclic (3,3,3,3-S4-Diol) and acyclic (3,3,3-S4-(COOH)₂ chelate systems.
- Discovered that traditional rhodium-105 radiolabeling techniques are not compatible with molecules that contain a carboxylic acid group.

Reportable Outcomes:

Presentations

Carroll, V; Wycoff, D; Sieckman, G; Hoffman, T; Jurisson, S; "Synthesis of a ^{105}Rh tetrathioether bombesin molecule for prostate cancer therapy" 243rd National Meeting of the American Chemical Society: San Diego, CA, March 25 – 29, 2012

Manuscripts

Carroll, V; Demoin, D; Hoffman, T; Jurisson, S; *Inorganic Chemistry in Nuclear Imaging and Radiotherapy: Current and Future Directions*. Radiochimica Acta, 2012. **100**: p. 653 – 667

Carroll, V; *Development of a Rhodium Tetrathioether Bombesin Analogue and Investigation of Cyclic and Acyclic Ligand systems for $^{105}\text{Rh(III)}$* . Ph. D. Thesis, Department of Chemistry. 2013, University of Missouri. p. 94

Carroll, V; Wycoff, D; Sieckman, G; Gallazzi, F; Hoffman, T; Jurisson, S; *Synthesis of a Rh tetrathioether bombesin analogue and impact of labeling conditions on chelate systems with pendant carboxylate groups*, (in preparation)

Degrees Obtained

Ph.D. in Chemistry, University of Missouri, May 2013

Employment Opportunities Received

Postdoctoral Scholar, University of California San Francisco, Department of Radiology and Biomedical Imaging, Mentor: Dr. David Wilson

Conclusion:

We have successfully synthesized a novel rhodium bombesin conjugate, $[\text{RhCl-S4-8Aoc-BBN(7-14)NH}_2]^+$. In vitro evaluation indicates high affinity for PC-3 human prostate cancer cells however low radiochemical yields of a single $[\text{}^{105}\text{Rh-S4-8Aoc-BBN(7-14)NH}_2]^+$ species on the radiotracer scale may preclude its usefulness as a radiotherapeutic agent. The pendant carboxylic acids were thought to be useful for maintaining reasonable hydrophilicity and thus clearance through the renal system. However, pendant carboxylic acids resulted in both complexes with a coordinated carboxylate and in esterification, the latter

is particularly a problem at the radiotracer level where acidic ethanolic reaction conditions are needed. In place of an acyclic bifunctional chelate (BFC) system with a pendant carboxylic acid, it is recommended to change the direction of research in future studies to investigate cyclic BFC's or acyclic BFC's with a methyl ester or methyl ether pendant group. This will prevent the formation of ethyl esters during radiolabeling.

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6. Goswami, N., et al., *Rhodium-105 tetrathioether complexes: radiochemistry and initial biological evaluation*. *Nuclear Medicine and Biology*, 1999. **26**(8): p. 951-957.